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Assessment of oxidative damage in the primary mouse ocular surface cells/ stem cells, in response to, Ultraviolet C (UV-C) damage

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Question	**Response**
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Mangalore
Sep 23, 2019

From:

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To,
Dr Phillip Steindel, PhD
Review Editor
Journal of Visualized Experiments (JOVE)

Subject: Submission of the revised manuscript **JoVE59924 - [EMID: 8f513a08842ce83b]-Revision 3**

Dear Dr Phillip Steindel,

Greetings!

I thank you for having given us the opportunity to revise the JOVE manuscript and providing us with sufficient deadline extensions. Now, we have revised the manuscript, as per the reviewer's suggestions. Please find our point-wise justifications, as well as, rebuttal towards the "Editorial Production Comments" and "Reviewer's comments".

Response to Editorial Production Comments
gg

Sr No	**Editorial Production Comments**	**Our responses**
1	0:01, 0:07, 0:10-1:35, 3:42, 4:54, 5:48, 7:35, 7:39-9:12, 12:43-end - There are still black borders on the left and right sides of the frame at these points. Some are very slight. Others are very visible. In either case, the white background at these points should be extended to fill in these black borders and create a unified background. This is the third time we are mentioning this issue; the next submission needs to demonstrate that you can address this comment.	We are genuinely apologetic for being unable to understand the black border issue. However, we have now tried resolving it, and no black border is visible in our system. Please let us know, if the black border is still visible at your end and the solution for the same.

2	3:45-4:49 - The narration audio still needs to be rerecorded. The loudness of the recording is causing audio distortion.	The clip has now been re-recorded.
3	11:58-12:40 - The introductory interview statement is now synchronized. However, the conclusion statement is not. The audio appears to be a few frames ahead of the video. This needs to be corrected, or the interview needs to be rerecorded.	Thank you for the suggestion. We have now corrected the same. We have also replayed the video several times and observed that the audio and video are in sync.
4	Please cite Table 3 in the manuscript outside of the Table Legends section.	Done. Please see the in-text citation highlighted in green colour on Page 10/14 in the manuscript.

TITLE:

Assessment of Oxidative Damage in the Primary Mouse Ocular Surface Cells/Stem Cells in Response to Ultraviolet-C (UV-C) Damage

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KEYWORDS

UV-C; primary ocular stem cells; Reactive oxygen species (ROS); 2',7'-dichlorofluoresceindiacetate (DCFDA); Live/dead cell detection, UV-C damage

SUMMARY:

This protocol demonstrates the simultaneous detection of reactive oxygen species (ROS), live cells, and dead cells in live primary cultures from mouse ocular surface cells. 2',7'-Dichlorofluoresceindiacetate, propidium iodide, and Hoechst staining are used to assess the ROS, dead cells, and live cells, respectively, followed by imaging and analysis.

ABSTRACT:

The ocular surface is subjected to regular wear and tear due to various environmental factors. Exposure to UV-C radiation constitutes an occupational health hazard. Here, we demonstrate the exposure of primary stem cells from the mouse ocular surface to UV-C radiation. Reactive oxygen species (ROS) formation is the readout of the extent of oxidative stress/damage. In an experimental in vitro setting, it is also essential to assess the percentage of dead cells generated due to oxidative stress. In this article, we will demonstrate the 2',7'-dichlorofluoresceindiacetate (DCFDA) staining of UV-C exposed mouse primary ocular surface stem cells and their quantification based on the fluorescent images of DCFDA staining. DCFDA staining directly corresponds to ROS generation. We also demonstrate the quantification of

dead and live cells by simultaneous staining with propidium iodide (PI) and Hoechst 3332 respectively and the percentage of DCFDA (ROS positive) and PI positive cells.

INTRODUCTION:

The ocular surface (OS) is a functional unit mainly composed of the outer layer and glandular epithelia of cornea, lachrymal gland, meibomian gland, conjunctiva, part of the eye lid margins and innervations that transduce signals[1]. The transparent dome shaped corneal layer focuses light onto the retina. This avascular tissue is composed of cellular components such as epithelial cells, keratocytes, and endothelial cells and acellular components such as collagen and glycosaminoglycans[2]. The area is drained by tears that also supply most of the nutrients. The anatomical position of the OS compels it to be in direct contact with the external environment, often exposing it to various harsh components such as bright light, microbes, dust particles and chemicals. This factor predisposes the OS to physical injuries and makes it prone to various diseases.

Oxidative stress is caused due to the disequilibrium between the production of reactive oxygen species (ROS) and the endogenous antioxidant defenses mechanisms[3]. ROS are classified into reactive molecules and free radicals, both of which are derived from molecular oxygen (O_2) through mitochondrial oxidative phosphorylation[4]. The former group is composed of non-radical species such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and the latter includes species such as superoxide anions (O_2^-), and hydroxyl radicals ($^\bullet OH$), among others. These molecules are by-products of normal cellular processes and their roles have been implicated in important physiological functions such as signal transduction, gene expression, and host defense[5]. An enhanced production of ROS is known to be generated in response to factors such as pathogen invasion, xenobiotics, and exposure to ultra violet (UV) radiation[4]. This overproduction of ROS results in oxidative stress that leads to the damage of molecules such as nucleic acids, proteins, and lipids[6].

Natural sunlight, the most predominant source of UV radiation, is composed of UV-A (400–320 nm), UV-B (320–290 nm), and UV-C (290–200 nm)[7]. An inverse correlation between the wavelength and spectral energies has been reported. Although natural UV-C radiations are absorbed by the atmosphere, artificial sources such as mercury lamps and welding instruments emit and, therefore, constitute an occupational hazard. Symptoms of exposure to eyes include photokeratitis and photokeratoconjunctivitis[8]. Production of ROS is one of the major mechanisms of inflicting UV induced cellular damage[9]. In the current study, we demonstrate the detection of ROS using the 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) staining method in mouse primary ocular surface cells/stem cells exposed to UV-C. The green fluorescence was captured using fluorescent microscopy. Cells were counter-stained with two dyes, Hoechst 33342 and red propidium iodide, to stain the live and dead cells, respectively.

PROTOCOL:

The experiment was performed on primary ocular cells/stem cells derived from the Swiss albino mouse eye. The use of animals for harvesting the eyes for this experiment was approved by the

Institutional Animal Ethical Committee, Yenepoya (Deemed to be University) (IEAC approval number, 6a/19.10.2016).

1. Preparations of reagents

NOTE: The derivation of primary cells/stem cells from the mouse ocular surface is beyond the scope of this protocol. Hence, we demonstrate the UV-C exposure doses, reagent preparation for assessing ROS, live and dead cells and their quantification. Please refer to **Table 1** for the respective volumes of the reagents (10% fetal bovine serum, DCFDA, Hoechst and propidium iodide stock solutions to be added for obtaining the final staining solution).

1.1. Prepare a stock solution of 10 mM DCFDA by dissolving 25 mg of DCFDA powder in 5.13 mL of DMSO. Aliquot 250 µL each in amber colored 1.5 mL tubes and store at -20 °C.

1.2. Prepare a stock solution of 10 mg/mL (16.23 mM solution) Hoechst 33342 by dissolving the entire contents of the 25 mg vial in 2.5 mL of deionized water. Make aliquots of 100 µL in amber colored microcentrifuge tubes and store at 2-6 °C for up to 6 months. For longer term storage, store at -20 °C.

1.3. Prepare a stock solution of 1 mg/mL propidium iodide in deionized water, aliquot 1 mL each in amber colored 1.5 mL tubes, and store at 4 °C.

2. **Cell plating and UV-C radiation treatment**

2.1. Before plating, dissociate the mouse primary ocular surface cells isolated in our laboratory (unpublished results; such cells are a mixture of corneal epithelial, stromal cells and keratocytes) using a gentle cell dissociation agent (**Table of Materials**).

2.2. Plate 0.2 x 10^6 mouse primary ocular surface cells in 35 mm 0.2% basement membrane matrix coated cell culture dishes in 2.5 mL of complete media. Incubate overnight at 37 °C in a 5% CO_2 and humidified incubator.

NOTE: Complete media for culturing primary cells from the mouse ocular surface is comprised of DMEM high glucose containing 20% FBS, 1% Pen-strep, 1% Glutamax, 1% non-essential amino acid (NEAA), 1% sodium pyruvate, and 0.1% β-mercaptoethanol.

2.3. Before exposing the cells to various doses of UV-C, discard the maximum volume of media and allow only a thin layer of media (~500 µL) to remain in contact with the cells, just enough to cover them.

2.4. Take the dishes, one at a time to the UV-C source/chamber (the lower chamber of a hybridization oven/UV cross linker; **Table of Materials**). Place the dish into the chamber and remove the lid of the dish. The open lid position of the dish ensures that the cells receive the maximum UV-C dose during the UV-C exposure.

2.5. Expose the cells to different grades/doses of UV-C: 1 J/m^2, 100J/m^2, 1,000 J/m^2 and 10,000 J/m^2.

2.6. After the UV-C exposure, replace the lid of each of the dishes immediately and remove them from the UV-C source chamber.

2.7. Bring each of the dishes to the laminar air flow hood and top up each of the dishes with 2 mL of fresh complete media.

2.8. Incubate the cells for 3 h in a 37 °C CO_2 incubator. Three hours of incubation post UV-C exposure is optimal to visualize and quantify the early effects.

3. Preparation of live-cell staining media

3.1. Prepare the staining media fresh during the last 15 min of the 3 h cell incubation post UV-C exposure.

3.2. Pre-warm 10 mL of the staining media containing 10% FBS-DMEM supplemented with 1% Pen-Strep to 37 °C.

3.3. Add 5 µL of 10 mM DCFDA; 5 µL of 10 mg/mL Hoechst solution and 200 µL of 1 mg/mL propidium iodide. The final concentrations of DCFDA, Hoechst and PI are 5 µM, 5 µg/mL and 20 µg/mL, respectively, in the 10 mL of staining media.

4. DCFDA staining of UV-C exposed mouse primary ocular cells

4.1. After 3 h of incubation of UV-C exposed mouse primary ocular cells at various doses, aspirate the media from the 35 mm dishes.

4.2. Replenish with 2 mL of freshly prepared DCFDA staining media to each of the dishes gently from the sides.

4.3. Incubate the cells with the staining media for 15 min in the dark in a 37 °C CO_2 incubator for live-cell staining.

5. Viewing of DCFDA (ROS), Hoechst and PI stained cells

5.1. After the completion of incubation, discard the staining media.

5.2. Add fresh complete media to the cells and observe the cells under an inverted fluorescent microscope/cell imager (**Table of Materials**). Photograph the desired fields: bright-field, blue fluorescence, red fluorescence, green fluorescence.

NOTE: The blue fluorescently stained cells are the nuclei, the green fluorescence is for ROS generating cells and the red fluorescence indicates the PI positive dead cells.

6. Quantification of stained cells (Hoechst-Blue, PI-Dead and Green-ROS) using imaging techniques

6.1. Export the images captured under the inverted fluorescent microscope/cell imager to ImageJ for quantification.

6.2. Open each of the images one at a time, using each channel (i.e., blue (Nuclei/Hoechst), green (ROS), red (Dead/PI positive)) sequentially, for counting. Start from the unexposed control and sequentially move to 1, 100, 1,000 and 10,000 J/m^{-2}.

6.3. Count the cells using the cell counting tool marked as a cross in the software menu for each of the fields [blue positive (Hoechst positive; nuclei), red positive (PI positive; dead cells); green positive (ROS)] in each of the images corresponding to each of the treatments.

6.4. Count by clicking on each of the specific signals in each of the fields. For example, clicking on the blue/Hoechst stained nuclei will give the total number of nuclei in a given field.

6.5. Calculate the results as the **percentage of cell death by UV damage** (number of PI positive cells x 100 divided by the number of Hoechst positive cells) and the **percentage of ROS production by UV damage** (number of DCFDA positive cells x 100 divided by the number of Hoechst positive cells).

REPRESENTATIVE RESULTS

DCFDA is a colorless dye that is a chemically reduced form of fluorescein used as an indicator for detecting ROS in cells. This dye gets trapped inside cells and is easily oxidized to fluorescent dichlorodihydrofluorescein (DCF), which emits a green fluorescence. This fluorescence can be detected using fluorescent microscopy. The cells can be visualized and correlated with ROS accumulation as follows: (i) live cells without ROS emit high blue fluorescence; (ii) live cells with ROS accumulation emit high blue fluorescence with low green fluorescence; and (iii) dead cells with ROS accumulation emit low blue fluorescence with high red and high green fluorescence.

Control cells and cells exposed to 1 J/m^2 of UV-C did not exhibit DCFDA/ROS and PI positive cells. The UV-C unexposed control showed nuclear staining only as indicated by the Hoechst staining (**Figure 1**). However, no PI or DCFDA positive cells were seen in the UV-C unexposed control cells (**Figure 1**).

Cells exposed to 100 J/m^2 of UV-C exhibited low ROS and PI positive cells. Primary cells from the mouse ocular surface upon exposure to UV-C at a dose of 100 J/m^2 showed about 5% co-staining of DCFDA and PI, thereby, indicating the formation of ROS and cell death in 5% of the cells (**Figure 1**).

Cells exposed to 1,000 J/m^2 of UV-C exhibited 60%–70% ROS and PI positive cells. Primary cells from mouse ocular surface upon exposure to UV-C at a dose of 1,000 J/m^2 showed about 70% co-staining of DCFDA and PI, thereby, indicating the formation of ROS and cell death in 70% of the cells (**Figure 1**).

Cells exposed to 10,000 J/m^2 of UV-C exhibited 100% ROS positive cells and ~100% cell death. The highest UV-C dose (10,000 J/m^2) when exposed to the mouse ocular primary cells resulted in 100% cell death (PI positive cells) and ROS formation (DCFDA staining), thereby indicating the highest lethality of this particular UV-C dose (**Figure 1**).

Cells treated with 1 J/m^2 of UV-C radiation did not show any accumulation of ROS and the percentage of live cells in this dose was comparable with that of the control cells. Cells demonstrated a significant amount of cell death and ROS accumulation at 100 J/m^2. The highest amount of ROS accumulation and cell death was observed in cells treated with 10,000 J/m^2 of UV-C radiation.

The quantified results (percentage of ROS generation and percentage of cell death as per the formulae given under section 6.3) were plotted in the form of a bar graph (**Figure 2**). The X-axis represents the UV-C doses while the Y-axis represents the percentage of cells. The green bar represents the percentage of ROS, and the red bars represent the cell death at different doses of UV-C radiation (**Figure 2**). The percentage of ROS and dead cells were of the order of 0%, 0%, 10%, 70% and 100% at the UV-C doses: unexposed control, 1 J/m^2, 100 J/m^2, 1,000 J/m^2 and 10,000 J/m^2, respectively (**Figure 2**).

FIGURE AND TABLE LEGENDS:

Figure 1: Composite live-cell images of the mouse ocular surface primary cells exposed to various doses of UV-C (Unexposed control, 1, 100, 1,000, 10,000 J/m^2). These images were captured under different filters: bright-field (for cell morphology), blue (Hoechst nuclear stain), green (ROS generation) and red (propidium iodide stained dead cells).

Figure 2: Bar graphs showing the quantification results obtained after calculating the percentage of ROS generation and percentage of dead cells upon exposure of primary mouse ocular surface cells to various doses of UV-C radiation. The X-axis represents the UV-C doses, while the Y-axis represents the percentage of cells.

Table 1: Reagents required for the preparation of staining solution.

Table 2: Troubleshooting.

DISCUSSION:

The DCFDA staining method described here enables the visualization of ROS in mouse primary ocular live cells treated with UV-C radiation. An advantage of this staining method is that it also allows the researchers to study the immediate effects of UV-C (3 hours post UVC exposure) on the live cells and their simultaneous enumeration for the percentage of ROS positive, as well as,

dead cells. Moreover, as the staining method is used on the live cells, the cells can be further incubated in the same media for a longer time (several days) for the study of delayed effects of UV-C radiation. Hence, this staining method makes it possible to visualize the ROS generation (green) and simultaneously distinguish the live, apoptotic and dead cell populations in live cells under an inverted fluorescence microscope. However, under certain circumstances, despite the anticipation of obtaining ROS positive and PI positive cells, the visualization can fail. For such instances, troubleshooting is suggested (**Table 2**).

This protocol must be carried out accurately to obtain optimal results. Optimal results indicate a correlation of the green fluorescent signal emitted/ROS generated as an accurate readout of the specific UV-C dose induced DNA damage. In other words, for carrying out this step, the volume of media that is in contact with the cells during the UV-C exposure should not be so much so that the UV-C dose fails to elicit the required damage. Hence, it is essential to keep a minimal volume of media, say 500 µL per 35 mm dish, just enough to cover the cells during the UV-C exposure at all the specified doses. Secondly, the volume of media should not be so little that the cells dry up during UV-C exposure. Finally, immediately after the exposure of the cells to the UV-C, the cells should be topped up with fresh media to the optimal volume (say 2 mL), in order to further avoid any damages and ROS generation.

One limitation of this technique is the type of ROS generated. Other additional assays must be performed to detect the specific ROS type in response to the UV-C dose ranges. Such assays include the assessment of the intracellular hydroxyl radical ($^{\bullet}OH$), intracellular superoxide radicals, intracellular reactive nitrogen species, mitochondrial hydroxyl radicals, mitochondrial superoxides, and hydrogen peroxide in live cells using commercially available kits. Another limitation of this technique is the detectability of the percentage ROS generation at doses below 10 J/m^2 and above 10,000 J/m^2. Apparently, no ROS positive cells were visible when the primary cells/stem cells from the mouse ocular surface were exposed to UV-C doses less than 10 J/m^2. On the contrary, 100% ROS positive cells were visible when the cells were exposed to UV-C dose above 10,000 J/m^2. Hence, other assays (e.g., enzyme analysis, measurement of oxidative stress markers such as 8-hydroxydeoxyguanosine (8-OHdG) (DNA damage marker), and western blot analysis of DNA damage proteins at various doses) might be useful to understand the extent/type of cellular/molecular damages at various levels. A third limitation of this technique is the correlation of the UV-C doses to the ROS generated across various cell types. This needs to be validated.

Currently, DCFDA kits are available commercially for ROS detection either using microscopy or flow cytometry[10,11]. However, such kits are expensive and cannot be afforded by research laboratories in resource restricted countries. Hence, this protocol is very useful with an efficiency akin to the commercially sold methods. Secondly, we have incorporated the propidium iodide dead cell stain along with the DCFDA/ROS generation. Hence, live cell monitoring of ROS positive and dead cells can be carried out simultaneously using this method.

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DISCLOSURES:

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Figure-1

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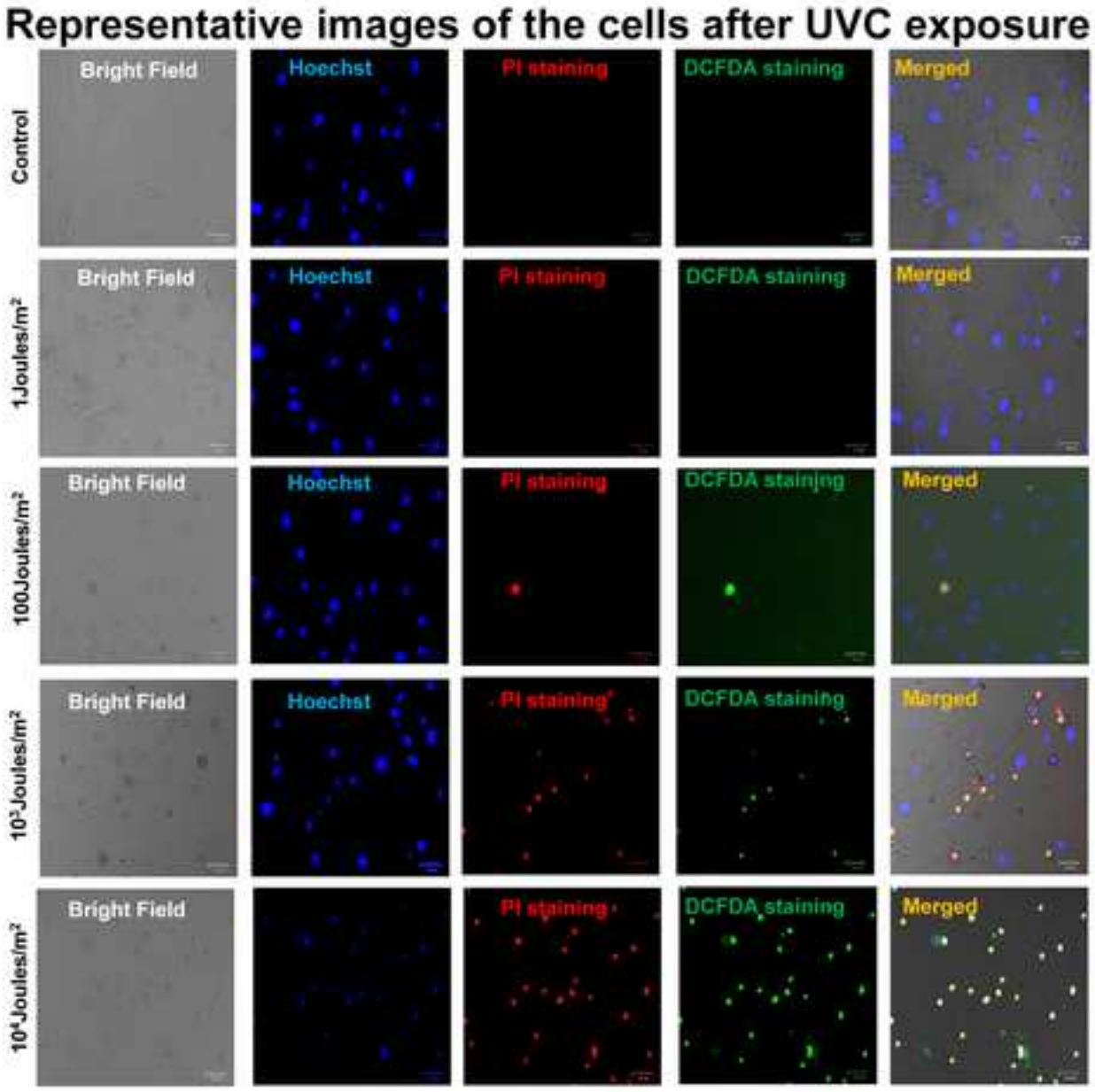
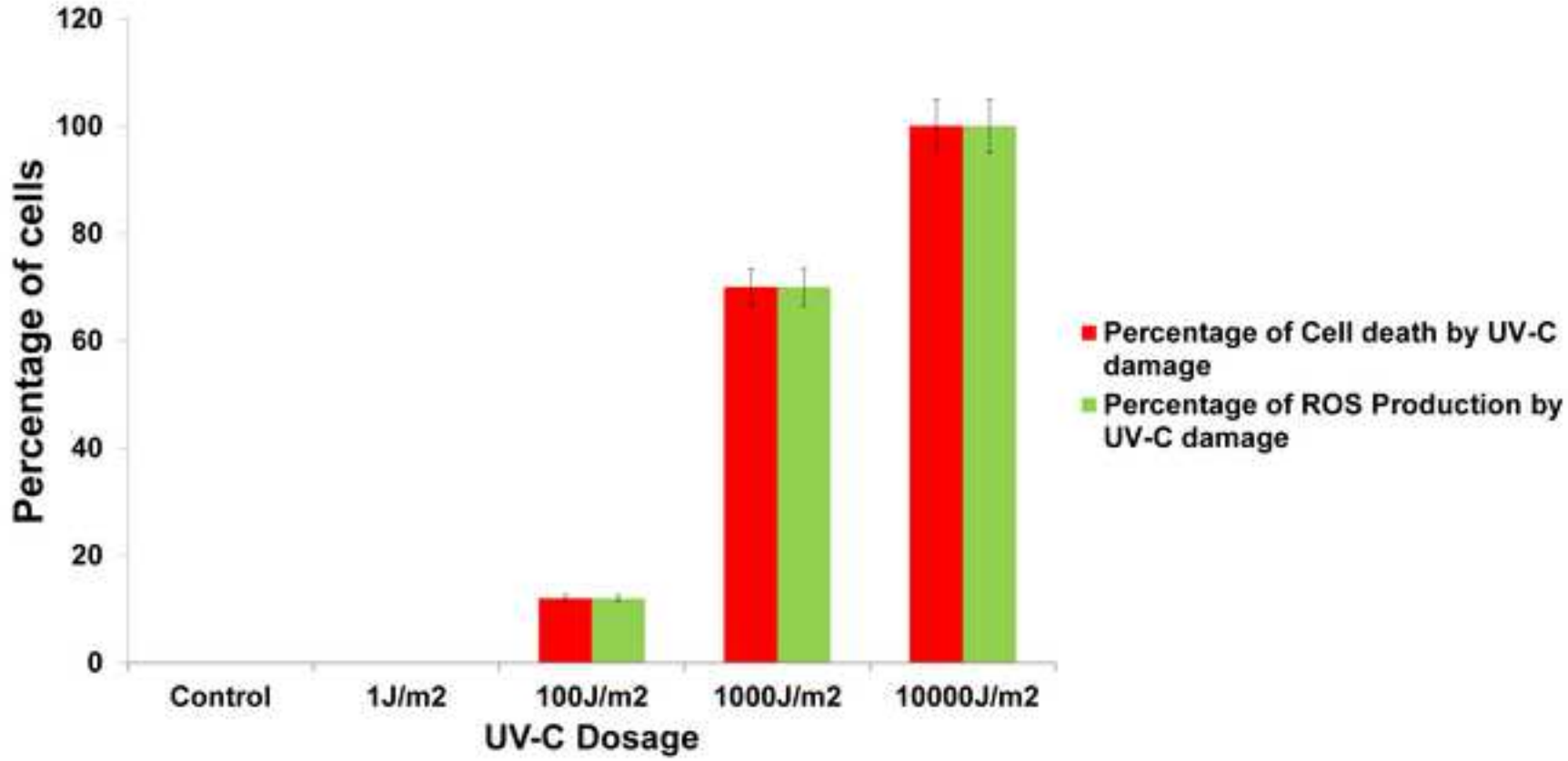


Figure 2



Component	Volume
10% Fetal Bovine Serum containing DMEM	9790 µL
DCFDA stock solution	5 µL
Hoechst 33342 stock solution	5 µL
Propidium iodide stock solution	200 µL

Problem	Probable reasons
No or very little ROS or PI positive cells are stained when the cells were treated with high to highest UV dose	i. Use of an old staining solution. ii. Overconfluent culture dish resulting in salvage of UVC damage by the cells.

Troubleshooting	Comments
i. Use freshly prepared staining solution. ii. Plate only 0.2 million cells in each 35 mm cell culture dish and never allow the cells to grow for more than 12 h before exposing them to UVC doses.	i. Earlier prepared staining solution leads to improper staining of the cells. ii. Overconfluent cells can salvage of UVC damage, and hence no or little ROS and PI positive cells will be visualized.

Table of Materials

Name of Material/Equipment	Company	Catalog Number
2', 7' –dichlorofluorescein diacetate(DCFDA)	Sigma	D6883
Cell culture dish (35 mm)	Eppendorf	SA 003700112
DMEM High Glucose	HiMedia	AT007
Fetal Bovine Serum, EU Origin	HiMedia	RM99955
GlutMax	Gibco, Thermo Fisher Scientific	35050061
HL-2000 Hybrilinker	UVP	
Hoechst 33342	Sigma	B2261
Matrigel	Corning	
MEM Non-Essential Amino Acids (100X)	Gibco, Thermo Fisher Scientific	11140050
Penicillin-Streptomycin (Pen-Strep)	Gibco, Thermo Fisher Scientific	15140122
Propidium Iodide	Sigma	P4170
TryplE Express	Thermo Fisher Scientific	
ZOE Fluorescent Cell Imager	Bio-rad	

Comments/Description

2',7'-Dichlorofluorescein diacetate is fluorogenic probe and is permeable to cells. It is used for quantification of reactive oxygen species.

Sterile dishes for culturing the cells.

Most widely used cell culture media, contains 4500 mg/L of glucose.

One of the most important components of cell culture media. It provides growth factors, amino acids, proteins, fat-soluble vitamins such as A, D, E, and K, carbohydrates, lipids, hormones, minerals, and trace elements.

Used as a supplement and an alternative to L-glutamine. It helps in improving cell viability and growth.

Hybridization oven/UV cross linker

Hoechst stain is permeable to both live and dead cells. It binds to double starnded DNA irrespective of wether the cell is dead or alive.

Basement membrane matrix

Used as a supplement to increase the cell growth and viability.

Penicillin and streptomycin is used to prevent the bacterial contamination in culture.

Fluorescent dye which is only permeable to dead cells. It binds with DNA and helps in distinguishing between live and dead cells.

Gentle cell dissociation agent

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Date:	Feb 28, 2019

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Mangalore
Sep 23, 2019

From:

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To,
Dr Phillip Steindel, PhD
Review Editor
Journal of Visualized Experiments (JOVE)

Subject: Rebuttal letter for the submission of the revised manuscript **JoVE59924 - [EMID: 8f513a08842ce83b]-Revision 3**

Dear Dr Phillip Steindel,

Greetings!

I thank you for having given us the opportunity to revise the JOVE manuscript and providing us with sufficient deadline extensions. Now, we have revised the manuscript, as per the reviewer's suggestions. Please find our point-wise justifications, as well as, rebuttal towards the "Editorial Production Comments" and "Reviewer's comments".

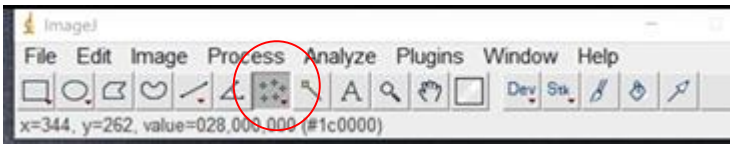
Response to Editorial Production Comments
gg

Sr No	Editorial Production Comments	Our responses
1	0:01, 0:07, 0:10-1:35, 3:42, 4:54, 5:48, 7:35, 7:39-9:12, 12:43-end - There are still black borders on the left and right sides of the frame at these points. Some are very slight. Others are very visible. In either case, the white background at these points should be extended to fill in these black borders and create a unified background. This is the third time we are mentioning this issue; the next submission needs to demonstrate that you can address this comment.	We are genuinely apologetic for being unable to understand the black border issue. However, we have now tried resolving it, and no black border is visible in our system. Please let us know, if the black border is still visible at your end and the solution for the same.

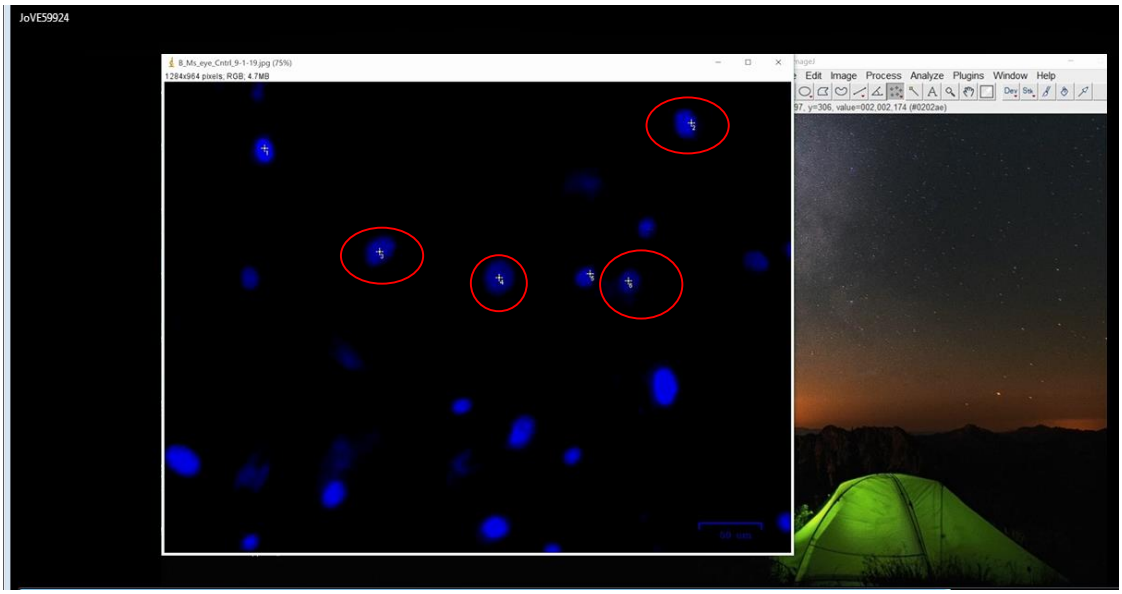
2	3:45-4:49 - The narration audio still needs to be rerecorded. The loudness of the recording is causing audio distortion.	The clip has now been re-recorded.
3	11:58-12:40 - The introductory interview statement is now synchronized. However, the conclusion statement is not. The audio appears to be a few frames ahead of the video. This needs to be corrected, or the interview needs to be rerecorded.	Thank you for the suggestion. We have now corrected the same. We have also replayed the video several times and observed that the audio and video are in sync.
4	Please cite Table 3 in the manuscript outside of the Table Legends section.	Done. Please see the in-text citation highlighted in green colour on Page 10/14 in the manuscript.

ImageJ steps

Step 6.2: Blue channel images



Step 6.3: Cell counting tool



Step 6.3: Clicking on the blue/Hoechst stained nuclei